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5.0 3T3 AND NHK NRU TEST METHOD DATA AND RESULTS

This section presents *in vitro* IC_{50} data generated by testing coded reference substances using the 3T3 and NHK NRU test method protocols. These IC_{50} values were used to evaluate the accuracy (also known as concordance)(see **Section 6**) and reliability (interlaboratory repeatability and reproducibility, intralaboratory reproducibility) (see **Section 7**) of these two *in vitro* cytotoxicity test methods. **Section 5.1** summarizes protocol modifications and revisions for each sequential phase of the validation study and examines whether such changes affected the data. **Section 5.2** provides the data used for assessing the accuracy and reliability of the 3T3 and NHK NRU protocols with a focus on PC data. **Section 5.3** summarizes the statistical approaches used for data evaluation and **Section 5.4** provides summaries of the acceptable 3T3 and NHK NRU test data for each reference substance (average IC_{50} for each laboratory/test method). **Section 5.5** describes the "lot-to-lot" consistency of the reference substances and adherence to GLP guidelines. **Section 5.6** provides the study timeline, **Section 5.7** describes availability of test data, and **Section 5.8** presents the solubility test data. The individual test data for both passing and failing tests (EXCEL® and PRISM® files) and summary spreadsheets are available on compact disk(s). Laboratory reports are also available on compact disk(s).

5.1 3T3 and NHK NRU Test Method Protocols

The protocols for the 3T3 and NHK NRU test methods used during Phase III laboratory testing phase are a result of modifications and revisions of the *Guidance Document* (ICCVAM 2001b) protocols and the optimization of the protocols used in the laboratory evaluation phases (Phases Ia and Ib) and the laboratory qualification phase (Phase II). **Figure 1-2** provides an outline of the study phases, as well as identifying where repeated observations were carried out to permit protocol evaluation and comparison. The following sections address the modifications of the protocols used in each phase and how those modifications affected each data set (**Section 2** details the similarities and differences between the two test method protocols).

5.1.1 Phase Ia: Laboratory Evaluation Phase

During Phase Ia, each testing laboratory established an historical database for the positive control chemical, sodium lauryl sulfate (SLS). No reference substances were tested in this phase. Ten concentration-response experiments were performed, with no more than two experiments/day, and the resulting data were used to calculate the acceptable response limits for use in Phase Ib testing.

Section 2.6.1 summarizes issues that occurred during this phase and addresses protocol changes made after the initiation of Phase Ia. The specific changes for both protocols are summarized here along with the impact the change had on the test data. Changes made in the protocols during Phase Ia were included in the Phase Ib protocols.

Protocol Changes and Impact on the Data

- *NR Dye Crystals:* Reduced the NR dye concentration for both cell types. No subsequent tests failed due to NR crystal formation and no apparent impact on the data was detected.
- *3T3 Cell Growth*: Modified cell culture conditions for 3T3 cells to improve cell growth characteristics. No apparent impact on the data was detected.
- *NHK Cell Growth (96-well plates):* Removed the cell culture-refeeding step performed prior to the reference substance application. SLS IC_{50} data were similar whether the cells were refed or not refed. The change in the protocol did not produce any observable impact on the data.
- *NHK Cell Growth (in culture flasks)*: FAL coated the culture flasks with fibronectin-collagen prior to seeding thawed cells. No apparent impact on data was detected.
- *OD Limits*: Eliminated the VC OD value range. The SMT accepted data from tests that were out of the OD range if all other criteria were met. Test data were not adversely affected by relaxing this criterion.
- *Dilution Factor*: The SMT accepted data generated using dilution factors other than the recommended 1.47 for definitive tests if all other test acceptance criteria were met. The use of smaller dilution factors generally increased the

number of points between 10 - 90% viability and the precision of the IC_{50} calculation was improved.

5.1.2 Phase Ib: Laboratory Evaluation Phase

The purpose of Phase Ib was to determine whether the protocol revisions from Phase Ia were effective in improving intra- and inter-laboratory reproducibility and to determine whether the laboratories could obtain reproducible results when testing coded reference substances of various toxicities. Three coded reference substances representing the full range of toxicity were tested in Phase Ib: arsenic trioxide (high toxicity), propranolol (medium toxicity), and ethylene glycol (low toxicity). Since Phase Ib was still part of the laboratory evaluation phase, the SMT decided that testing just three substances was sufficient and the substances did not need to represent all GHS toxicity categories. Each substance was tested at least once in a range finding experiment and then in three acceptable definitive tests performed on three different days.

Section 2.6.2 summarizes the technical challenges that arose during this phase and addresses protocol changes made after initiation of Phase Ib. This section (**5.1.2**) describes the specific changes for the 3T3 and NHK NRU protocols along with the impact the changes had on the test data.

Protocol Changes and Impact on the Data

- *NR Dye Crystals*: Reduced the concentration of NR in the 3T3 test method. The OD values and SLS IC_{50} data were similar in four exploratory experiments regardless of the NR concentration or the NRU incubation time tested. The elimination of NRU crystals reduced the background OD values.
- *OD Range*: Used new OD ranges only for guidance (e.g., target values to assess adequate cell growth) for the remainder of the study. This increased the number of tests that met the acceptance criteria. Data were not adversely affected by the removal of this criterion.
- *SLS IC_{50} Range*: Expanded the acceptance criterion range for the SLS IC_{50}. This allowed additional positive control tests to meet the acceptance criteria and

thereby qualifying additional definitive tests as acceptable since they would meet acceptance criteria and not fail simply because the PC failed.

5.1.3 Phase II: Laboratory Qualification Phase

The results of Phase II determined whether the protocol revisions from Phase Ib were effective in improving intra- and inter-laboratory reproducibility and whether the laboratories could obtain reproducible results when testing a larger set of substances covering a wider range of physical/chemical characteristics and toxicities than tested in Phase Ib. Nine coded reference substances were analyzed: aminopterin, cadmium chloride, chloramphenicol, colchicine, lithium carbonate, potassium chloride, 2-propanol, sodium fluoride, and sodium selenate. These substances were common to the RC (with the exception of sodium selenate) and were chosen because they fit the RC millimole regression line (i.e., were within the acceptance intervals of the regression line). The RC is a database of acute oral LD_{50} values for rats and mice obtained from RTECS® and IC_{50} values from *in vitro* cytotoxicity assays using multiple cell lines and cytotoxicity endpoints for chemicals with known molecular weights (Halle 1998). Sodium selenate, the non-RC chemical, was chosen because of its high toxicity. Besides aminopterin, there were no other reference substances in the highest toxicity category that were within the RC millimole regression acceptance intervals. Each substance was tested at least once in a range finding experiment and then in three acceptable definitive tests performed on different days during this phase.

Sections 2.6.2 and **2.6.3** summarize the technical issues that arose during this phase and address NRU protocol changes made prior to Phase II. This section (**5.1.3**) describes the additional changes for both 3T3 and NHK NRU protocols along with the impact the changes had on the test data.

Protocol Changes and Impact on the Data

- *Blank Wells*: Added reference substance to blank wells of the test plate. There was no apparent impact on test data.
- *VC OD Range*: Eliminated the VC OD range as an acceptance criterion. There was no apparent impact on test data.

- *Harmonization of Laboratory Techniques*: Made revisions to the Phase II protocols as a result of the harmonization training by the testing laboratories (see **Section 2.6.2**). There was no apparent impact on test data for IIVS and ECBC but FAL data quality was improved.
- *3T3 Cell Seeding Density*: Added a range of cell seeding densities to be used by the laboratories. No apparent impact on data was detected during this phase.
- *NHK Cell Growth from Cryopreservation*: Eliminated the use of fibronectin-collagen coating and 80-cm^2 flasks for initial propagation of NHK cells. FAL achieved better cell growth, obtained lower IC_{50} values for the PC, and achieved better agreement of the mean SLS IC_{50} values compared to the other laboratories.
- *Volatile Substances*: Added CO_2 permeable plate sealer use for control of volatility in subsequent experiments (identified by cross contamination of the control wells). The use of plate sealers for volatile substances was incorporated into the Phase III protocols.
- *Hill Function*: Relaxed the Hill function criteria. Some tests that did not meet the original criterion were accepted by the SMT after determining that even though the curve fit was not optimum, the curve adequately conveyed the toxicity of the substance.
- *Unusual Dose Response*: Revised the Hill function calculation to address substances that produced a dose-response for which toxicity plateaued before reaching 0% viability. This allowed for calculation of a more precise IC_{50} value for such substances.
- *Positive Control IC_{50} Range*: Expanded the SLS IC_{50} acceptable range, which resulted in additional tests in Phase II being acceptable. Expanding the PC range reduced the number of retests of reference substances and thereby qualifying additional definitive tests as acceptable since they would meet acceptance criteria and not fail simply because the PC failed.

5.1.4 Phase III: Main Validation Phase

The purpose of Phase III was to generate high quality *in vitro* cytotoxicity data using the 3T3 and NHK NRU test methods with optimized test method protocols. Sixty coded reference substances were tested (see **Table 5-3**); 46 of these were RC chemicals that covered a broad range of toxicity. The substances in Phase III spanned all five GHS toxicity categories and included unclassified substances. Each substance was tested at least once in a range finding experiment and then in three acceptable definitive tests performed on different days. **Tables 5-3** and **5-4** provide summary data for the Phase III substances.

Section 2.6.4 addresses protocol changes made before initiation of Phase III. This section (**5.1.4**) describes the specific changes for both 3T3 and NHK NRU protocols along with the impact the changes made on the test data.

- *Prequalification of NHK Culture Medium*: Included a protocol for prequalifying NHK culture medium and supplements. This prevented the participating laboratories from using medium and supplements that did not provide adequate growth characteristics for NHK cells.
- *Stopping Rule for Testing*: Added this rule for chemicals that were insoluble (i.e., solubility < 200 μg/mL) or could not achieve adequate toxicity over the concentration range tested; this rule allowed testing to end for chemicals that produced no IC_{50} data within three definitive tests. Chemicals that could not be adequately tested by one or more laboratories are presented in **Table 5-1**. In all three laboratories, carbon tetrachloride could not be adequately tested in either 3T3 or NHK cells while methanol could not be adequately tested in 3T3 cells.
- *Acceptable Range for Dose-Response Data Points:* Modified the test acceptance criterion for the number of data points required on the toxicity curve. Changed from requiring a minimum of two points (at least one point > 0% and ≤ 50% viability and at least one point > 50% and < 100% viability) to one point > 0% and < 100% viability if the smallest practical dilution factor was used (i.e., 1.21) and all other test acceptance criteria were met. This reduced the number of failed experiments without reducing the quality of the IC_{50} data.

- *R^2 Acceptance Criteria*: Rescinded the R^2 criterion for the fit of the Hill function. The SMT determined that the R^2 criterion was best used to characterize the reference chemical response curve shape rather than to establish a criterion for test acceptability. This reduced the number of failed experiments without reducing the quality of the IC_{50} data.
- *PC Acceptance Criteria*: Modified the PC acceptance criterion for Hill function fit.
- *Hill Function Analysis*: Altered the PRISM® template for the Hill function analysis to perform calculations for IC_x values in two ways: (1) constraining Bottom parameter to zero and (2) fitting the Bottom parameter. As a result of the changes and efforts by the laboratories to use dilution schemes that captured the entire dose-response, very few tests in Phase III had $R^2 < 0.9$.
- *Biphasic Dose Response*: This aspect was added to the Phase III protocol so that the Study Directors could make a decision about analyzing data from reference substances with biphasic dose-responses (See **Section 2.6.3**).

Table 5-1 Reference Substances Affected by Stopping Rule

Reference Substance[1]	Testing Stopped -- No Data					
	3T3 NRU Test Method			NHK NRU Test Method		
	ECBC	FAL	IIVS	ECBC	FAL	IIVS
Carbon tetrachloride	X	X	X	X	X	X
Disulfoton		X				
Gibberellic acid		X				
Methanol	X	X	X	X		
1,1,1-Trichloroethane	X				X	X
Valproic acid			X			
Xylene	X	X		X	X	

[1]Substances that did not provide adequate cytotoxicity
ECBC: Edgewood Chemical Biological Center
FAL: FRAME Alternatives Laboratory
IIVS: Institute for In Vitro Sciences

5.2 Data Obtained to Evaluate Accuracy and Reliability

This section first presents the acceptable PC data from each laboratory for each phase of the validation study and then presents the reference substance data for each phase. All test data, both acceptable and unacceptable, are available on compact disk upon request. Accuracy (concordance) and reliability assessments are provided in **Section 6** and **Section 7**, respectively.

5.2.1 PC Data

A summary of the acceptable SLS IC_{50} data used to calculate quality control acceptance limits for each experiment, by laboratory, to use in subsequent study phases, are shown in **Table 5-2.**

Phase Ib Acceptance Limits

The acceptance limits for the SLS IC_{50} for Phase Ib testing were calculated using the Phase Ia data. The data sets from each laboratory were examined for outliers using the method of Massey and Dixon (1981), but none were identified. The acceptance limits for the SLS IC_{50} values for each laboratory and test method were mean ± 2 SD since the SD is more commonly used as a range than the 95% confidence limits.

Phase II Acceptance Limits

The IC_{50} values from the SLS tests from Phases Ia and Ib were used to calculate laboratory-specific and test method-specific quality control acceptance limits for Phase II. Phase Ib tests with SLS IC_{50} values outside of the acceptance limits were considered acceptable if they met all other test acceptance criteria. For any day during which there was more than one SLS test (for each test method and laboratory), the IC_{50} values were averaged to better reflect day-to-day variation and avoid overweighting the overall mean with values from an individual day. Extreme values were tested and removed if they were outliers at the 99% level and the remaining values were used to calculate the mean ± 2.5 SD as the acceptance limits. The acceptance limits were expanded from 2 SD in Phase Ib to 2.5 SD for Phase II to allow for the fact that the limits tend to get narrower as more data are collected.

Table 5-2 Positive Control (SLS) Data by Phase

Study Phase	ECBC				FAL				IIVS			
	Mean IC50 (µg/mL)	Standard Deviation (µg/mL)	Acceptance Limits	N	Mean IC50 (µg/mL)	Standard Deviation (µg/mL)	Acceptance Limits	N	Mean IC50 (µg/mL)	Standard Deviation (µg/mL)	Acceptance Limits	N
3T3												
Ia[1]	38.3	4.71	28.8 – 47.7	15	42.3	8.56	25.2 – 59.5	25	40.9	3.19	34.5 – 47.3	12
Ib[2]	41.3	5.99	26.4 – 56.3	12	43.2	4.68	31.5 – 54.9	17	42.1	3.40	33.6 – 50.6	13
II[3]	41.2	4.20	30.8 – 51.6	29	45.9	7.50	27.2 – 64.7	36	40.6	3.50	31.8 – 49.3	21
III[4]	41.6	3.41	NA	65	41.1	6.23	NA	26	41.5	3.74	NA	22
NHK												
Ia[1]	4.03	1.32	1.40 – 6.67	15	7.45	3.07	1.34 – 13.6	18	3.68	0.555	2.57 – 4.79	30
Ib[2]	3.65	0.98	1.22 – 6.10	11	5.35	2.32	0[a] – 11.1	15	3.57	0.59	2.10 – 5.04	17
II[3]	3.59	1.41	0.07 – 7.11	22	3.20	1.05	0.57 – 5.82	15	3.78	0.73	1.94 – 5.61	26
III[4]	3.03	0.75	NA	57	3.45	0.90	NA	35	3.12	0.53	NA	20

[1]Values generated from Phase Ia data for PC acceptance criterion for Phase Ib; Acceptance limits = Mean ± 2 X standard deviation
[2]Values generated from Phases Ia and Ib data for PC acceptance criterion for Phase II; Acceptance limits = Mean ± 2.5 X standard deviation
[3]Values generated from Phases Ia, Ib, and II data for PC acceptance criterion for Phase III; Acceptance limits = Mean ± 2.5 X standard deviation
[4]Values generated from Phase III data.
[a]Calculation of lower limits actually yielded negative concentrations, so lower limit was placed at 0 and later revised to 0.1 µg/mL
NA = not applicable
ECBC: Edgewood Chemical Biological Center
FAL: FRAME Alternatives Laboratory
IIVS: Institute for In Vitro Sciences

Phase III Acceptance Limits

The IC_{50} values from the SLS tests from Phases I and II were used to calculate laboratory-specific and test method-specific quality control acceptance limits for Phase III. The SLS IC_{50} values outside of the acceptance limits were considered acceptable if the tests met all other test acceptance criteria. For any day for which there was more than one SLS test (for each test method and laboratory), the IC_{50} values were averaged to better reflect day-to-day variation and avoid overweighting the overall mean with values from an individual day. ANOVA was used to compare the Phase Ia, Ib and II data within each laboratory. For phases that were not significantly different at $p < 0.05$, the IC_{50} data were used to calculate the mean ± 2.5 SD as the acceptance limits for Phase III. The only laboratory/test method that showed a significant difference between the phases was FAL using the NHK NRU test method ($p < 0.0002$). The difference was attributed to the changes in cell culture practices between Phases Ib and II (see **Section 5.1.3**). Thus, for the NHK data at FAL, only the Phase II SLS IC_{50} values were used to calculate the acceptance limits for Phase III.

The IC_{50} values from the SLS tests from Phase III are also presented in **Table 5-2**.

5.2.2 Reference Substance Data

All reference substance data from all laboratories are presented in **Appendix I**. **Tables 5-3**, **5-4**, and **5-5** and **Figures 5-1 a-f** (3T3) and **5-2 a-f** (NHK) provide summary data for all phases of the NICEATM/ECVAM validation study (see **Section 5.4**).

5.3 Statistical Approaches to the Evaluation of 3T3 and NHK NRU Data

Statistical approaches to data evaluation are reviewed in the following sections for each phase of the NICEATM/ECVAM validation study. **Section 2.2.3** discusses the endpoint measurements for the 3T3 and NHK NRU test methods. The mean OD values of the six replicate values (six wells [minimum of four] in the 96-well plate) per test concentration (eight concentrations/reference substance or PC) are used to determine relative cell viability by calculating the specific concentration's percentage of the mean NRU of all VC values on the same plate. The mean cell viability values generated from replicate wells for each concentration are used to plot a toxicity curve (percent viability versus concentration) and the IC_{50} value is determined from that curve.

5.3.1 <u>Statistical Analyses for Phase Ia</u>

The laboratories reported the IC_{50} results for SLS in μg/mL. The SMT used the results from the acceptable tests to calculate means and SDs for each test method at each laboratory.

Outlier Determination for Replicate Well Concentration Data

During a review of the six replicate well OD data for the same concentration of a reference substance, it was noted that extreme OD values sometimes occurred and that removal of these "outlier values" frequently improved the fit of the Hill function for the concentration cytotoxicity response curve. Concern was expressed that the outliers, if not excluded, may create so much noise that the true cytotoxicity response might be obscured although there was no discernable experimental reason for the outliers. Although it was recognized that removal of extreme values reduced reported variability and might have altered the mean value, an outlier test from Dixon and Massey (1981) was used to evaluate the consistency of replicate well data. The SMT manually applied the outlier test to the Phase Ia data when apparent extreme values were noted. If the extreme value was an outlier at the 99% level, it was excluded from the data set, and the IC_{50} was recalculated. All data are available in the data files provided by the laboratories, including the OD values in the excluded outlier value wells. The protocol acceptance requirement of a minimum of four test wells per reference substance concentration remained in effect.

Curve Fit Criterion

Upon visual review of the fit of the OD data to the Hill function curve, a curve fit criterion was implemented as a test acceptance criterion. The SMT considered the fit of the concentration-response curve to the Hill function to be acceptable when $R^2 > 0.9$. If $R^2 < 0.8$, then the fit was unacceptable and the data for that test was rejected. Curves with a fit of $0.8 < R^2 < 0.9$ were evaluated visually (for goodness of fit) and accepted if the SMT concluded that there were sufficient data points between 0 and 100% cytotoxicity and a reasonable shape to the curve to calculate a reasonably accurate IC_{50}. Each test with a curve fit in this range was analyzed individually (i.e., on a case-by-case basis) and no standard criterion was developed to pass/fail such results. [Note: The use of R^2 was reevaluated in Phases Ib and II and was eliminated as a test acceptance criterion for Phase III reference substances. An R^2 value ≥ 0.85 was maintained as a test acceptance criterion for the PC.] The R^2 criterion was implemented approximately two months after the laboratories completed Phase Ia testing.

Reproducibility Analyses for PC IC_{50} Values

To evaluate reproducibility of the IC_{50} values for the PC for each test method, within and between the laboratories, the SMT considered using the American Society of Testing and Materials (ASTM) Standard E691-99, Standard Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method (ASTM 1999). This method uses two statistics, h and k, to judge the consistency of means and variances between laboratories. Since a minimum of six laboratories is required for this type of analysis, the SMT decided that it could not be appropriately applied to three laboratories.

Therefore, the variability of the IC_{50} data obtained for each test method and laboratory for the PC was assessed using CV analysis and one-way analysis of variance (ANOVA). The CV was calculated by dividing the SD by the arithmetic mean IC_{50} value and then multiplying by 100. CV values were calculated for the acceptable tests within each laboratory. To compare the variation among laboratories, CV was calculated from the mean IC_{50} values from each laboratory. Although no criterion for acceptable CV was determined for this study, ECVAM has recently used CV < 30% as an acceptable CV range for both intra- and inter-laboratory reproducibility (Zuang et al. 2002; Fentem et al. 2001). ECVAM usually applies the criterion to the mean CV for all substances tested during the same phase. Although this CV range is intended to reflect an acceptable maximum for normal biological variability, the range is not supported by data.

For the ANOVA, IC_{50} values were first converted to mM units and then log-transformed to obtain normal distributions. One-way ANOVA was performed with SAS PROC GLM (SAS Institute 1999; see **Appendix R1** for example SAS code). To be conservative with respect to identifying laboratory differences, a significance level of $p < 0.01$ was used to test results between the laboratories.

5.3.2 Statistical Analyses for Phase Ib

Outlier Determination for Replicate Well Concentration Data

For consistency of replicate well concentration data, the SMT applied the same outlier test used for the Phase Ia data (Dixon and Massey 1981) when extreme OD values were noted. If the extreme value was an outlier at the 99% level, it was excluded from the data set, and the IC_{50} was recalculated. All data are available in the data files provided by the laboratories, including the OD values in the excluded outlier value wells.

Reproducibility Analyses for the Reference Substance IC_{50} Values

A one-way ANOVA and CV analyses were used to assess test method reproducibility within and across laboratories were performed as described in **Section 5.3.1**. When the ANOVA detected significant differences among the laboratories ($p< 0.01$), contrast analyses were performed to determine which laboratory was different from the others. The contrasts compared the results of each laboratory with those of the other two laboratories. A significant difference among the laboratories was indicated by $p < 0.01$.

5.3.3 Statistical Analyses for Phase II

Outlier Determination for Replicate Well Concentration Data

For consistency of replicate well concentration data, the outlier test from Dixon and Massey (1981) was incorporated into the EXCEL® templates used by the laboratories to collect and report data. Extreme values that were outliers at the 99% level were highlighted and the Study Director was offered the option of removing the value from subsequent calculations (for mean OD of the six replicates, % viability, IC_{50}, etc.).

Reproducibility Analyses for Reference Substance IC_{50} Values

CV values from the acceptable tests were used to calculate mean, SD, and CV for each substance/test method/laboratory as described in **Section 5.3.2**. Intra- and inter-laboratory reproducibility of IC_{50} data, by test method, for the reference substances tested in Phases II was also assessed using one-way ANOVA as described in **Section 5.3.2**.

Comparison of 3T3 and NHK NRU Test Results to the RC Millimole Regression

To compare the 3T3 and NHK NRU test results for the reference substances to those of the RC millimole regression, the IC_{50} values reported by the laboratories were transformed to mM units for the calculation of geometric mean IC_{50} values for each substance/test method/laboratory. The log geometric mean IC_{50} values were used with the RC LD_{50} values (see **Table 3-2**), after transformation to log mmol/kg units (see **Appendices J1** and **J3**), to calculate least squares linear regressions for each test method and laboratory. Each of these regressions was compared to the RC millimole regression using an F test with SAS PROC REG (SAS Institute 1999; see **Appendix R2** for example SAS code). An F test with a significance level of $p < 0.01$ was used to determine whether the joint comparison of slope and intercept indicated that the laboratory regressions were significantly different from the RC millimole regression.

5.3.4 Statistical Analyses for Phase III

Outlier Determination for Replicate Well Concentration Data

The laboratories used the outlier test at the 99% level (Dixon and Massey 1981) incorporated into the EXCEL® templates to test for outlier values among replicate well concentration data. The Study Director had the option of excluding the outliers from the data set, which were highlighted by the template, from subsequent calculations. All data are available in the data files provided by the laboratories, including the OD values in the excluded outlier value wells.

Reproducibility Analyses for the PC Data

A number of analyses were performed to determine whether the SLS IC_{50} values were reproducible over the duration of the study (i.e., across study phases). The SLS IC_{50} values used to access variability were somewhat different from those shown in **Table 5-2**. To get an assessment of the true variation of SLS IC_{50} values, the reproducibility analyses included IC_{50} values from SLS tests that failed the test acceptance criterion for the IC_{50} acceptance limits in **Table 5-2** that were determined for each laboratory and study phase. These SLS tests, however, passed all other test acceptance criteria. If more than one SLS test was performed in a single day (for each test method and laboratory), the IC_{50} values were averaged to determine a single IC_{50} for the day so that multiple results from a single day would not overly influence the average for each phase. CV analyses were performed as described in **Section 5.3.1** using the arithmetic mean IC_{50} values for each test method, laboratory, and study phase.

For the remaining analyses of reproducibility, the IC_{50} values were first log-transformed to obtain normal distributions. One-way ANOVAs were performed with SAS PROC GLM (SAS Institute 1999; see **Appendix R1** for example SAS code) for each test method using study phase and laboratory individually as explanatory variables. A significance level of $p < 0.01$ was used to test for a statistical difference among the laboratory and/or phase results. To determine whether there was a linear time trend for the SLS IC_{50} data, linear regression analyses using a least squares method were performed for each laboratory and test method using SAS PROC REG (SAS Institute 1999). Time was expressed as an index for each test. The index number of each test reflected its order of testing without respect to the time lapsing between tests. The slopes of the linear regressions were statistically significant if $p < 0.05$.

Reproducibility Analyses for the Reference Substance Data

CV and one-way ANOVA analyses were performed to assess the intra- and inter-laboratory reproducibility of the Phase III reference substance data as described in **Section 5.3.2**.

The geometric mean IC_{50} values were used to calculate least squares linear regression models after log transforming the data. Linear regressions were fit for each test method and laboratory using the log transformed reference LD_{50} values from **Table 4-2** in mmol/kg with log IC_{50} in mM. To detect differences between the laboratory regressions, two models were fit for each test method. The first model was a full model that included effects for laboratory and interactions. This model generated a regression line for each laboratory. The second model, the reduced model, assumed that one model fit all the laboratories. A goodness of fit F test was performed to compare the full and reduced models for the two regressions for each test method. A significance level of $p < 0.05$ was used to test whether the laboratory regressions were significantly different from one another.

Comparison of 3T3 and NHK NRU Test Results to the RC Regression

The laboratory regressions for each test method were combined using the log geometric mean of the geometric mean IC_{50} values from each laboratory and the reference log transformed LD_{50} in mmol/kg. Another linear regression was calculated using the log transformed IC_{50} and LD_{50} data from the RC for the 58 RC chemicals tested in the NICEATM/ECVAM validation study. The regression for the 58 RC chemicals was compared to the combined laboratory regressions for each test method using an F test to compare slope and intercept (simultaneously). A $p < 0.01$ was used to indicated whether the test method regressions were statistically different from the 58 chemical RC regression.

To assess accuracy of the regression models and the NRU test methods, the LD_{50} predictions of the RC millimole regression and two additional regressions developed in **Section 6.2** were used to assign predicted GHS acute oral toxicity category categories (see **Section 6.3**). Accuracy was determined by calculating the proportion of chemicals for which the predicted GHS toxicity category matched the *in vivo* GHS toxicity category. The LD_{50} predictions from these regression models were also used to determine starting doses for acute systemic toxicity test method simulations for the purpose calculating animal use and animal savings using the NRU test methods. The simulation modeling methods and results for the UDP and ATC methods are described in **Section 10**.

5.4 Summary of Results

Table 5-3 the reference substance name, chemical class (classification based on the National Library of Medicine's Medical Subject Heading [MeSH]), summary IC_{50} data (arithmetic mean), standard deviations, and the number (N) of tests used to produce the values in the study for both *in vitro* NRU cytotoxicity test methods. Data are categorized alphabetically and by phase. The reference substance data are also shown on bar graphs in **Figures 5-1 a-f** (3T3) and **5-2 a-f** (NHK) and the reference substances are ranked by IC_{50} values (lowest value [most toxic] to highest value [least toxic]). The substances are divided into subgroups for ease of fit to the graph size. **Appendices I-1** through **I-4** provide all test data (IC_{50} values) from all laboratories for each cell type. **Tables 5-4** and **5-5** provide the geometric IC_{50} mean values for 3T3 and NHK (laboratories combined) and show the differences in the values in orders of magnitude. The correlation of the mean IC_{50} values for the 58 study reference substances common to the RC database vs the RC IC_{50} values is shown in **Figure 5-3** (3T3 NRU values) and **Figure 5-4** (NHK NRU values). **Table 5-7** contains summary data for the solubility studies performed by the laboratories. **Table 5-8** lists the reference substances that exhibited precipitate and/or volatility problems. **Appendix F** provides physical, chemical, and biological information for all 72 reference substances.

Table 5-3 3T3 and NHK NRU Test Method Summary IC_{50} Data from the Laboratories

Substance	Chemical Class[4]	3T3 NRU Test Method									NHK NRU Test Method								
		ECBC			FAL			IIVS			ECBC			FAL			IIVS		
		IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N
Phase Ia																			
Sodium lauryl sulfate (SLS)	Alcohol	38.6	3.8	12	44.8	4.7	21	40.9	3.2	12	4.11	1.4	13	6.64	2.1	14	3.63	0.5	29
Phase Ib																			
Arsenic III Trioxide	Arsenical	2.41	0.782	4	1.04	0.070	4	4.09	2.23	3	7.77	2.54	4	2.55	1.92	6	20.9	6.40	3
Ethylene glycol	Alcohol	18325	1658	4	31650	7453	4	25900	3081	3	38000	4681	3	49800	4371	3	40000	5341	4
Propranolol HCl	Alcohol	13.6	4.37	4	13.5	6.85	4	17.6	3.78	3	38.3	4.54	3	43.8	2.52	3	28.6	3.28	4
Phase II																			
Aminopterin	Heterocyclic	0.005	0.001	3	0.012	0.005	3	0.005	0.001	3	889	182	3	545	42.2	3	611	70.7	2
Cadmium II chloride	Cadmium compound	0.480	0.066	3	0.400	0.129	3	0.817	0.427	3	2.20	0.823	5	1.88	1.22	3	1.86	0.151	3
Chloramphenicol	Alcohol	55.3	12.4	4	273	82.2	4	156	27.9	3	318	142	3	414	182	4	367	79.7	3
Colchicine	Heterocyclic	0.021	0.002	4	0.093	0.042	3	0.028	0.0003	3	0.005	0.002	3	0.008	0.001	3	0.008	0.002	3
Lithium I carbonate	Lithium compound	564	67.6	3	NA	NA	NA	NA	NA	NA	411	119	3	486	95.7	3	535	31.6	3
Potassium I chloride	Potassium, chlorine compound	3352	468	4	3842	1198	5	3710	417	3	2560	432	3	2287	631	3	1990	161	3
2-Propanol (Isopropyl alcohol)	Alcohol	2610	240	2	3970	139	3	4110	161	3	5263	583	3	4273	1139	3	7087	480	3
Sodium I fluoride	Sodium, fluorine compound	61.3	5.55	3	96.1	17.7	3	82.0	5.81	3	48.7	6.92	3	39.7	9.61	3	53.7	6.82	4

Table 5-3 3T3 and NHK NRU Test Method Summary IC_{50} Data from the Laboratories

Substance	Chemical Class[4]	3T3 NRU Test Method									NHK NRU Test Method								
		ECBC			FAL			IIVS			ECBC			FAL			IIVS		
		IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N
Sodium selenate	Sodium, selenium compound	12.7	1.62	3	54.2	10.4	3	36.5	5.23	3	7.47	0.861	3	16.1	9.55	3	10.0	1.33	3
Phase III																			
Acetaminophen	Amide	40.8	9.12	3	66.2	23.0	3	43.4	11.4	3	558	80.7	3	447	83.7	3	571	79.0	3
Acetonitrile	Nitrile	6433	129	3	9690	5634	3	9330	1217	3	10868	7824	4	10153	1960	4	9290	413	3
Acetylsalicylic acid	Carboxylic Acid	646	61.5	3	1234	298	3	401	62.0	3	631	19.9	3	694	98.3	3	514	79.1	3
5-Aminosalicylic acid	Carboxylic Acid	1467	203	3	2070	334	3	1557	179	3	29.9	6.52	3	78.2	42.3	3	48.8	7.90	3
Amitriptyline HCl	Polycyclic	6.03	1.38	3	7.86	2.20	3	7.81	1.38	3	10.8	3.34	3	7.57	5.43	3	10.9	1.04	3
Atropine sulfate	Heterocyclic	54.1	29.6	3	133	41.1	3	70.0	5.7	3	85.4	10.5	3	104	88.2	3	83.2	21.0	3
Boric acid	Boron compound	1497	484	3	3987	693	3	1202	581	3	440	138	3	517	378	3	464	11.0	3
Busulfan	Alcohol	40.4	19.3	3	321	180	3	43.7	1.77	3	253	68.2	3	268	193	3	313	37.2	3
Caffeine	Heterocyclic	133	13.3	3	157	81.7	3	191	14.4	3	817	256	3	591	186	3	574	7.81	3
Carbamazepine	Heterocyclic	83.0	12.0	3	152	56.9	3	91.8	11.0	3	66.1	8.40	3	253	325	3	63.9	5.27	3
Carbon tetrachloride	Halogenated hydrocarbon	NA	NA	-	NA	NA	-	NA	NA	-	NA	NA	-	NA	NA	-	NA	NA	-
Chloral hydrate	Alcohol	151	15.6	3	241	25.1	3	170	19.9	3	140	34.2	3	159	50.1	3	112	1.73	3
Citric acid	Carboxylic acid	473	138	3	1148	143	4	865	160	3	526	82.4	3	312	51.6	4	433	22.3	3

Table 5-3 3T3 and NHK NRU Test Method Summary IC_{50} Data from the Laboratories

Substance	Chemical Class[4]	3T3 NRU Test Method									NHK NRU Test Method								
		ECBC			FAL			IIVS			ECBC			FAL			IIVS		
		IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N
Cupric sulfate pentahydrate	Sulfur compound	82.7	3.18	3	123	54.0	4	5.72	1.75	3	190	19.6	3	195	12.5	3	207	7.09	3
Cycloheximide	Heterocyclic	0.125	0.057	3	0.647	0.451	3	0.109	0.025	3	0.053	0.012	3	0.120	0.094	3	0.071	0.013	3
Dibutyl phthalate	Carboxylic acid	23.5	3.98	3	191	94.5	4	20.7	1.37	3	28.3	7.64	3	47.4	34.3	3	22.0	1.32	3
Dichlorvos	Organophos-phorous	9.83	3.42	3	32.8	2.07	3	18.3	2.09	3	8.56	2.28	3	12.4	3.74	3	12.2	0.416	3
Diethyl phthalate	Carboxylic acid	85.5	29.0	3	147	37.8	3	106	25.3	3	174	14.4	3	71.5	67.3	3	189	33.1	3
Digoxin	Polycyclic	351	137	3	892	319	3	317	67.9	2	0.0054	0.0007	3	0.0001	0.00002	3	0.0040	0.0003	3
Dimethyl-formamide	Amide	5343	515	3	5483	517	3	4900	183	3	9353	155	3	7817	100	3	6397	202	3
Diquat dibromide monohydrate	Heterocyclic	3.87	0.887	3	36.1	35.5	3	5.39	1.36	3	3.59	0.825	3	6.77	3.73	4	3.84	0.313	3
Disulfoton	Organophos-phorous compound	137	74.9	3	11200	NA	1	60.4	52.5	3	140	27.0	3	808	213	3	186	59.2	3
Endosulfan	Heterocyclic	5.27	3.01	3	15.2	11.9	4	3.61	1.53	3	3.44	0.573	3	1.42	0.701	4	2.19	0.437	3
Epinephrine bitartrate	Alcohol	51.5	6.16	3	63.4	6.63	3	63.4	1.91	3	115	10.8	3	81.7	28.4	3	75.0	12.2	3
Ethanol	Alcohol	5360	1754	3	8420	1205	3	6413	345	3	8290	390	3	12013	2286	3	10250	867	3
Fenpropathrin	Hydrocarbon	22.6	2.41	3	42.4	26.8	4	16.7	2.03	3	3.73	1.01	3	2.23	0.616	3	1.82	0.310	3
Gibberellic acid	Hydrocarbon	8027	908	3	NA	NA	-	7657	745	3	2850	402	3	2940	276	3	2807	121	3

Table 5-3 3T3 and NHK NRU Test Method Summary IC_{50} Data from the Laboratories

Substance	Chemical Class[4]	3T3 NRU Test Method									NHK NRU Test Method								
		ECBC			FAL			IIVS			ECBC			FAL			IIVS		
		IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N
Glutethimide	Heterocyclic	167	7.00	3	284	20.7	3	125	9.25	4	187	64.3	3	170	24.1	3	176	27.5	3
Glycerol	Alcohol	20000	2987	3	38878	28238	4	27833	10882	3	34267	15399	3	18023	8334	3	29033	4596	3
Haloperidol	Ketone	5.32	0.649	3	7.99	0.655	3	5.47	0.654	3	3.69	1.01	3	3.72	1.81	3	3.29	1.15	3
Hexachlorophene	Cyclic hydrocarbon	5.02	2.41	3	5.35	1.75	3	3.06	0.289	3	0.027	0.004	3	0.046	0.020	3	0.021	0.002	3
Lactic acid	Carboxylic acid	2943	315	3	3487	561	3	2790	259	3	1290	52.9	3	1320	60.8	3	1313	138	3
Lindane	Halogenated hydrocarbon	125	119	3	266	94.8	4	90.4	111	5	19.1	3.14	3	23.2	7.09	3	15.6	2.40	3
Meprobamate	Carboxylic acid	353	49.7	3	877	128	4	386	9.02	3	761	116	3	163	189	3	624	84.2	3
Mercury II chloride	Mercury compound	3.45	0.177	3	5.99	1.87	3	3.51	0.120	3	6.87	1.04	3	5.40	1.02	3	5.35	0.090	3
Methanol	Alcohol	NA	NA	-	NA	NA	-	NA	NA	-	NA	NA	-	1133	213	3	2100	226	3
Nicotine	Heterocyclic	272	65.3	3	412	136	3	450	54.7	3	94.3	24.7	3	134	78.4	3	112	27.7	3
Paraquat	Heterocyclic	21.3	7.29	3	24.9	16.5	3	23.7	15.2	3	48.3	6.03	3	96.6	37.2	3	53.4	5.52	3
Parathion	Organophos-phorous compound	22.7	12.1	3	141	98.7	4	22.0	4.94	3	34.0	10.0	3	31.2	11.9	3	29.0	8.34	3
Phenobarbital	Heterocyclic	634	134	3	726	255	3	476	111	4	693	180	3	360	95.5	3	381	69.9	3
Phenol	Phenol	50.2	10.9	3	104	24.8	3	58.1	6.78	3	59.1	21.4	3	93.2	5.97	3	80.8	5.12	3

Table 5-3 3T3 and NHK NRU Test Method Summary IC_{50} Data from the Laboratories

Substance	Chemical Class[4]	3T3 NRU Test Method									NHK NRU Test Method								
		ECBC			FAL			IIVS			ECBC			FAL			IIVS		
		IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N	IC_{50}[1] µg/mL	SD[2]	N
Phenylthiourea	Sulfur compound	30.1	19.8	3	239	65.8	3	89.0	21.9	3	363	58.0	3	401	83.6	3	272	71.7	3
Physostigmine	Carboxylic acid	28.2	14.9	3	37.8	1.93	3	20.4	6.71	4	164	5.51	3	212	238	3	139	8.74	3
Potassium cyanide	Potassium, nitrogen compound	15.3	3.76	3	159	81.9	3	18.9	0.950	3	29.3	6.90	3	89.0	100	3	16.9	2.21	3
Procainamide HCl	Amide	400	15.3	3	431	4.73	3	497	39.3	3	1480	200	3	1787	221	3	2027	229	3
Propylparaben	Carboxylic acid	20.9	3.33	3	51.8	14.8	3	17.1	2.10	3	18.1	2.42	3	18.6	2.84	3	13.8	1.21	3
Sodium arsenite	Arsenical	0.496	0.028	3	1.44	0.819	3	0.683	0.117	3	0.790	0.248	3	0.336	0.187	3	0.470	0.066	3
Sodium chloride	Sodium, chlorine compound	4790	233	3	4625	611	4	4877	457	3	3583	263	3	1118	1388	3	3470	300	3
Sodium dichromate dihydrate	Sodium, chromium compound	0.603	0.087	3	0.657	0.244	3	0.547	0.092	3	0.784	0.113	3	0.851	0.302	4	0.576	0.100	3
Sodium hypochlorite	Sodium, oxygen, chlorine compound	823	108	3	805	367	3	2005	872	4	1863	581	3	1243	576	3	1633	180	3
Sodium oxalate	Carboxylic acid	42.0	17.3	3	31.0	8.66	3	49.5	26.3	4	355	54.9	3	350	147	4	360	94.6	3
Strychnine	Heterocyclic	389	80.9	3	124	20.3	3	83.5	5.35	3	100	76.6	4	52.5	28.0	3	55.1	3.43	3
Thallium I sulfate	Metal	2.81	0.671	3	13.4	10.4	4	6.27	1.75	3	0.198	0.100	3	0.153	0.031	3	0.127	0.020	3

Table 5-3 3T3 and NHK NRU Test Method Summary IC_{50} Data from the Laboratories

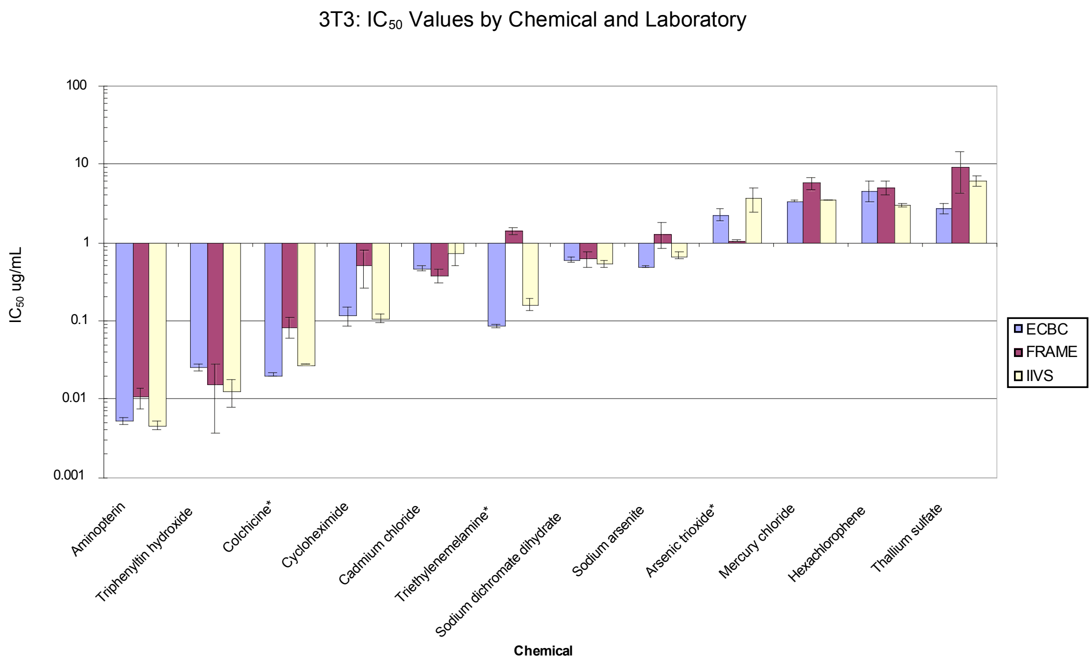
Substance	Chemical Class[4]	3T3 NRU Test Method									NHK NRU Test Method								
		ECBC			FAL			IIVS			ECBC			FAL			IIVS		
		IC_{50}[1] μg/mL	SD[2]	N	IC_{50}[1] μg/mL	SD[2]	N	IC_{50}[1] μg/mL	SD[2]	N	IC_{50}[1] μg/mL	SD[2]	N	IC_{50}[1] μg/mL	SD[2]	N	IC_{50}[1] μg/mL	SD[2]	N
Trichloroacetic acid	Carboxylic acid	762	99.1	3	1220	72.1	3	801	114	3	348	63.5	3	541	150	3	394	50.8	3
1,1,1-Trichloro-ethane	Halogenated hydrocarbon	41100	NA	1	21250	2357	3	9827	180	3	8137	591	3	NA	NA	-	NA	NA	-
Triethylene-melamine	Triazine	0.086	0.009	3	1.45	0.265	3	0.169	0.049	3	1.69	0.950	3	2.03	0.471	3	2.13	0.480	3
Triphenyltin hydroxide	Organo-metallic compound	0.026	0.004	3	0.026	0.021	3	0.015	0.008	3	0.021	0.007	3	0.007	0.007	3	0.011	0.003	3
Valproic acid	Carboxylic acid	547	67.1	3	1807	175	3	574	NA	1	468	116	3	702	160	3	430	71.5	3
Verapamil HCl	Amine	32.2	5.82	3	34.6	1.72	3	38.9	4.20	3	60.5	13.6	3	79.4	33.9	3	66.2	5.57	3
Xylene	Cyclic hydrocarbon	NA	NA	-	NA	NA	-	724	87.1	3	NA	NA	-	NA	NA	-	486	185	3

[1]Arithmetic mean
[2]Standard deviation
[3]Data are slightly different from that summarized in **Table 5-2** for Phase Ia. These data represent the acceptable tests after implementation of the R^2 acceptance criterion, while the data in **Table 5-2** represent acceptable tests prior to the implementation of the criterion.
[4]Chemical class assigned is based on the classification of the National Library of Medicine's Medical Subject Heading (MeSH), http://www.nlm.nih.gov/mesh/meshhome.html
NA = not available; IC_{50} values could not be generated (see footnotes in **Appendix J**)

Figure 5-1 3T3 NRU IC_{50} Values by Reference Substance and Laboratory

(Substances are grouped from lowest mean IC_{50} value (aminopterin) to highest mean IC_{50} value (ethylene glycol).

a



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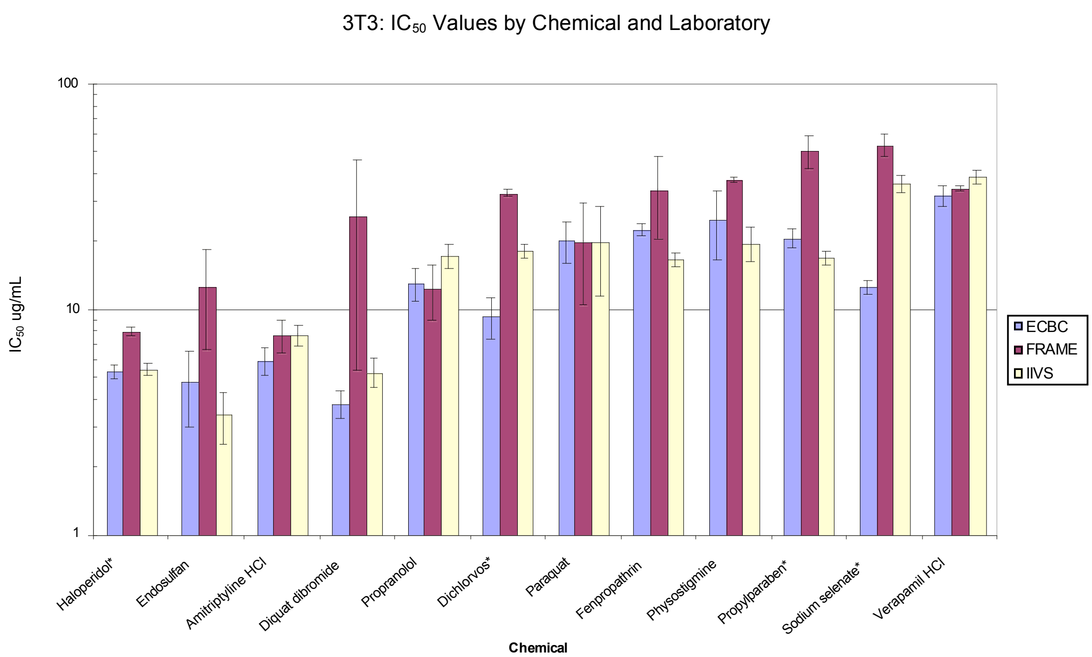

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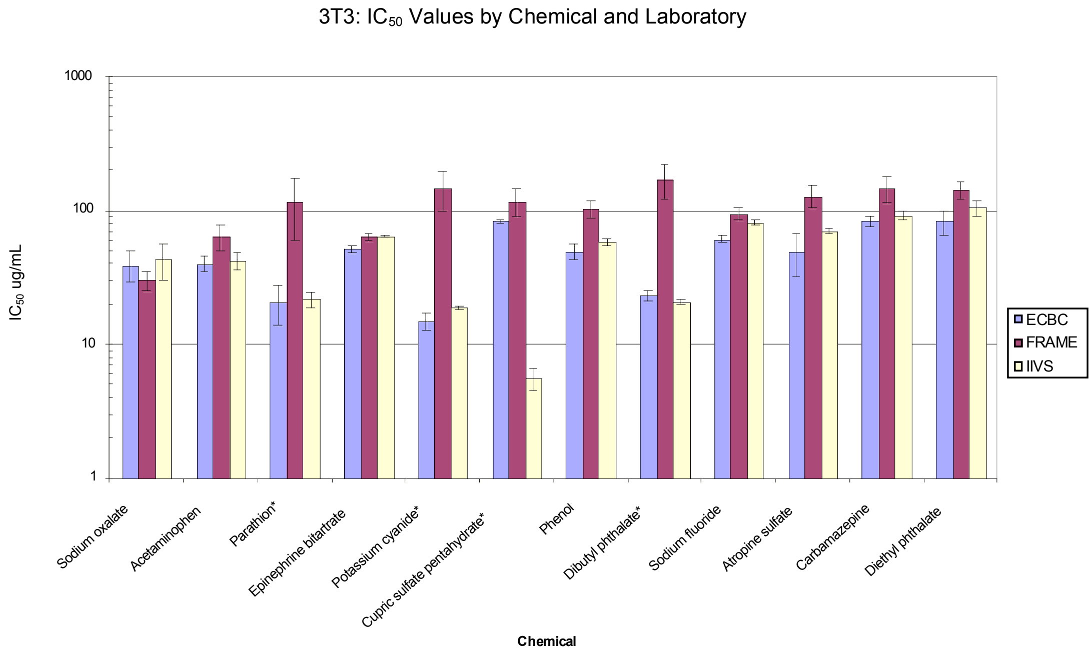
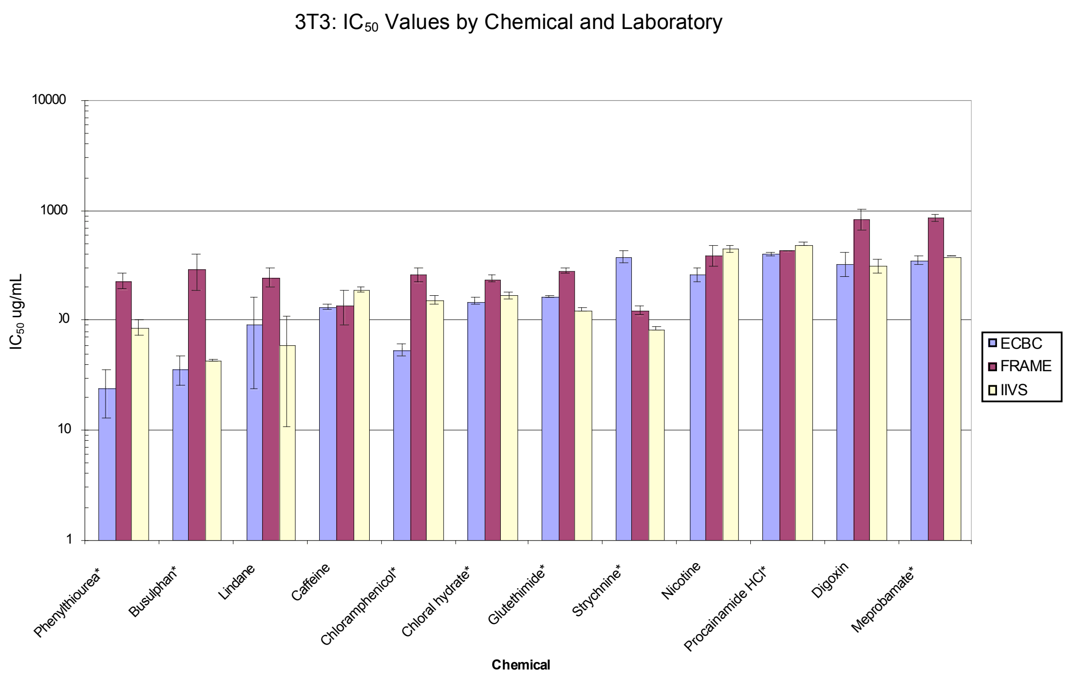
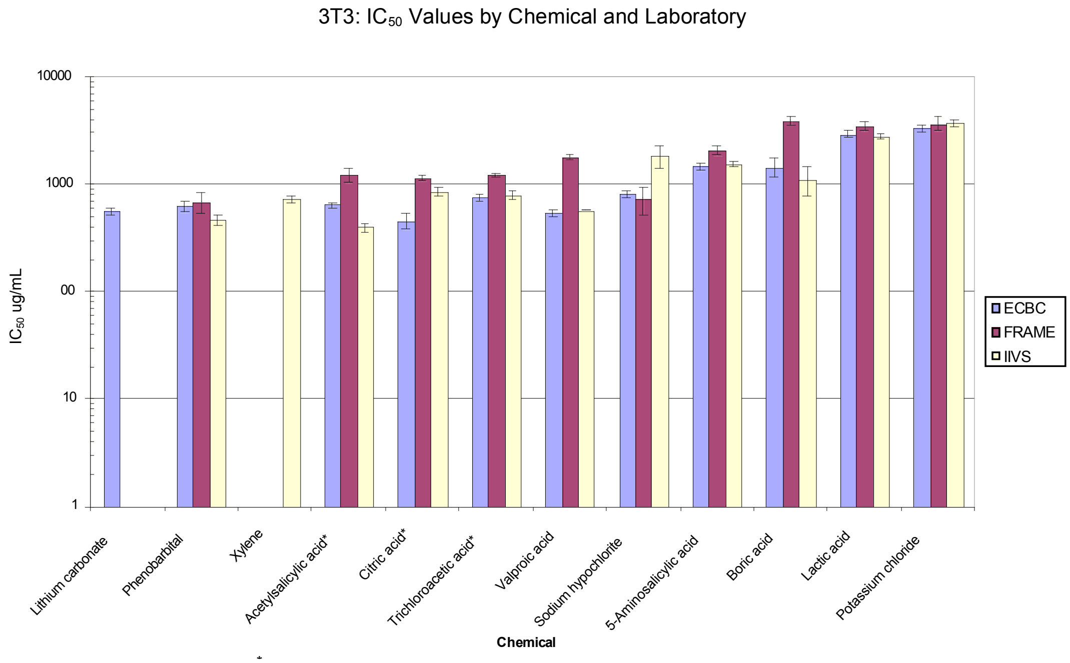

3T3: IC_{50} Values by Chemical and Laboratory
IC_{50} ug/mL
1000
100
10
1
Sodium oxalate
Acetaminophen
Parathion*
Epinephrine bitartrate
Potassium cyanide*
Cupric sulfate pentahydrate*
Phenol
Dibutyl phthalate*
Sodium fluoride
Atropine sulfate
Carbamazepine
Diethyl phthalate
Chemical
ECBC
FRAME
IIVS

d


3T3: IC_{50} Values by Chemical and Laboratory
IC_{50} ug/mL
10000
1000
10
1
Phenylthiourea*
Busulphan*
Lindane
Caffeine
Chloramphenicol*
Chloral hydrate*
Glutethimide*
Strychnine*
Nicotine
Procainamide HCl*
Digoxin
Meprobamate*
Chemical
ECBC
FRAME
IIVS

e



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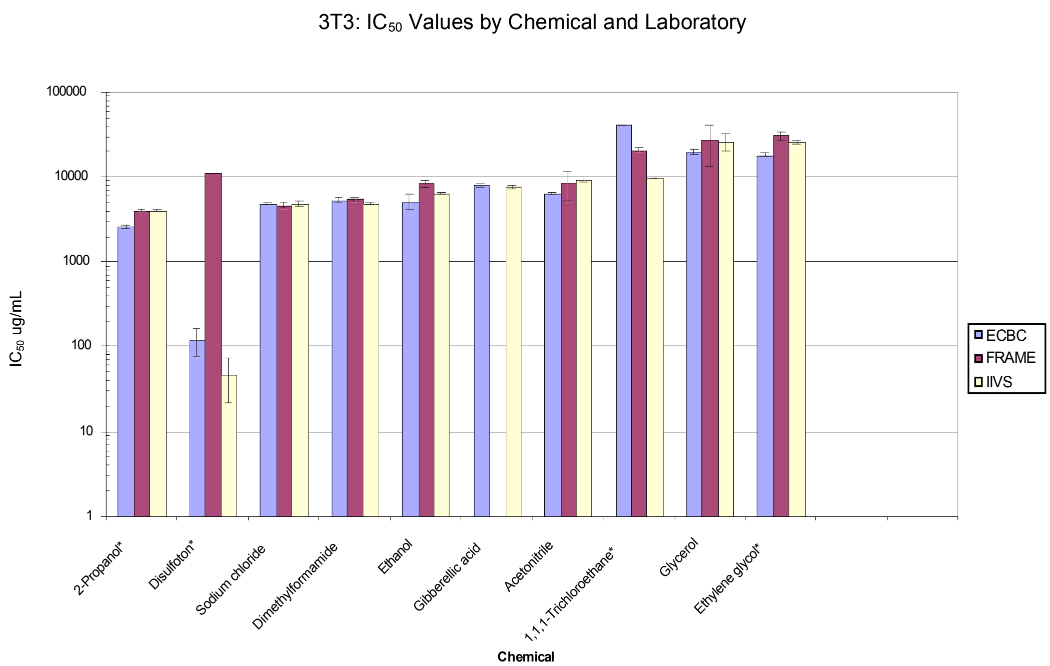
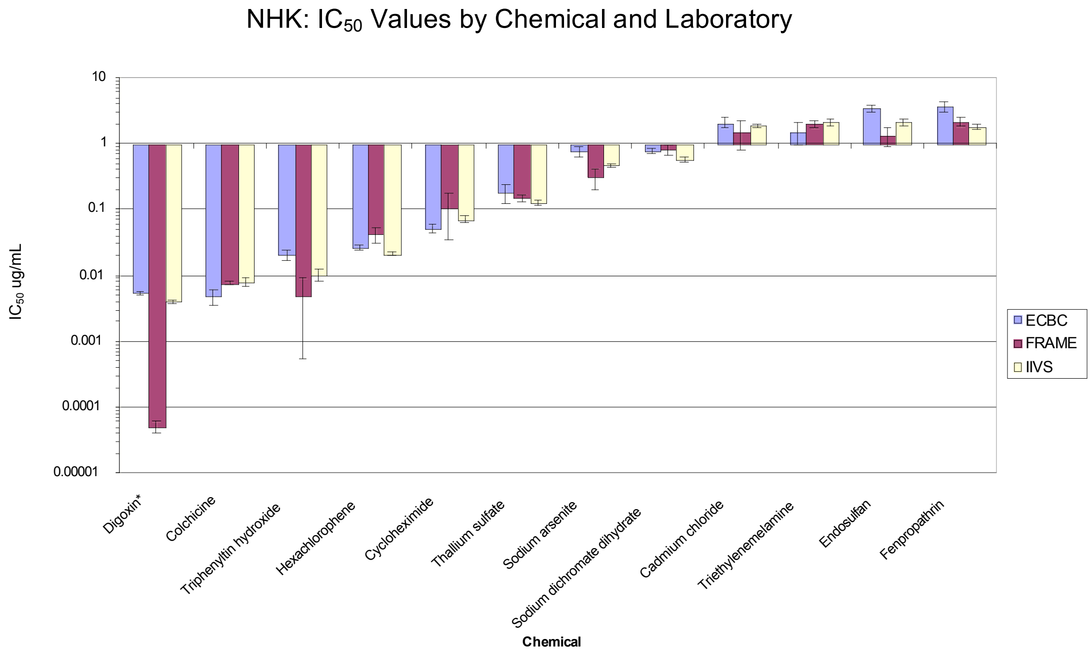


*Represents a chemical where the standard ANOVA indicates a significant difference in IC_{50} values between laboratories. Bars represent mean IC_{50} from each laboratory in μg/mL. Log IC_{50} values used to allow multiple data sets on each graph. Error bars show the standard deviation.

Figure 5-2 NHK NRU IC_{50} Values by Reference Substance and Laboratory
(Substances are grouped from lowest mean IC_{50} value (digoxin) to the highest mean IC_{50} value (ethylene glycol).

a



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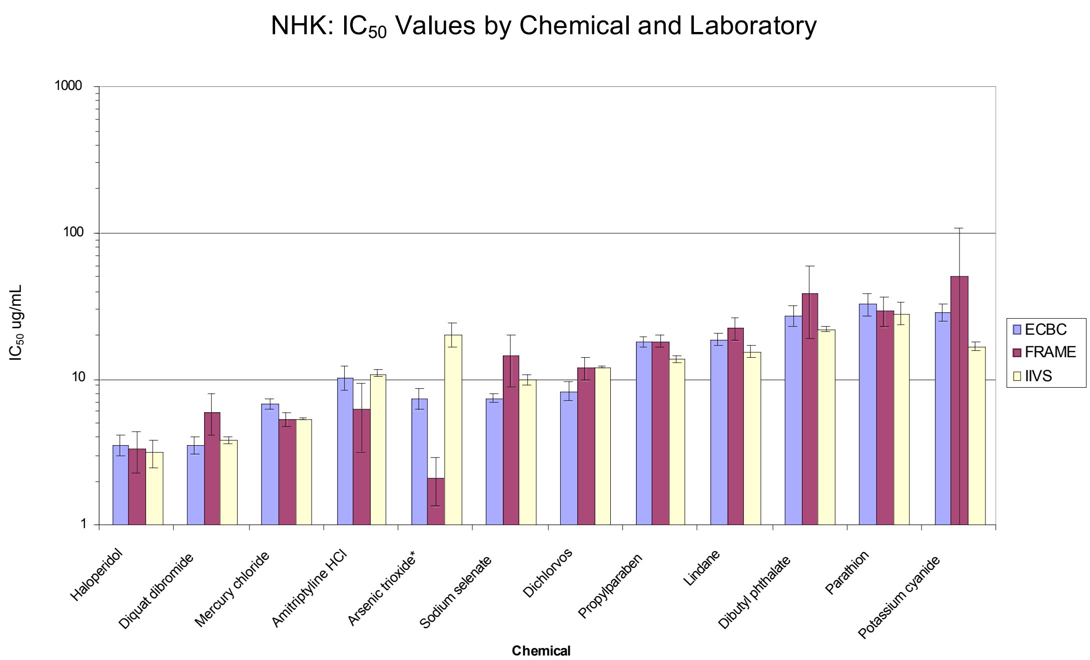

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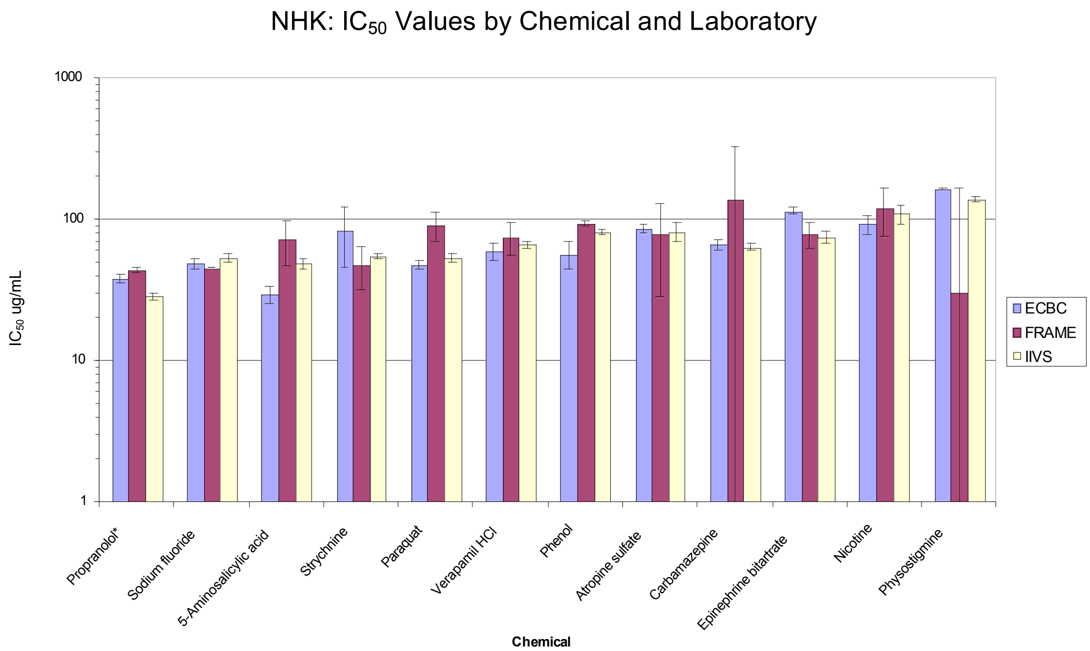
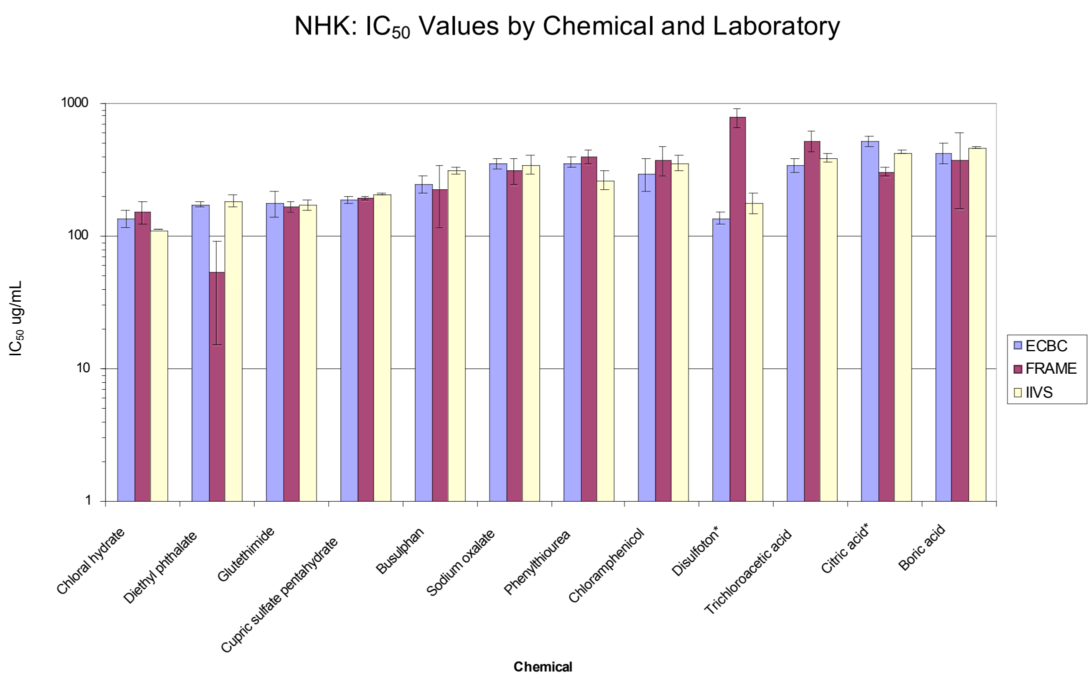
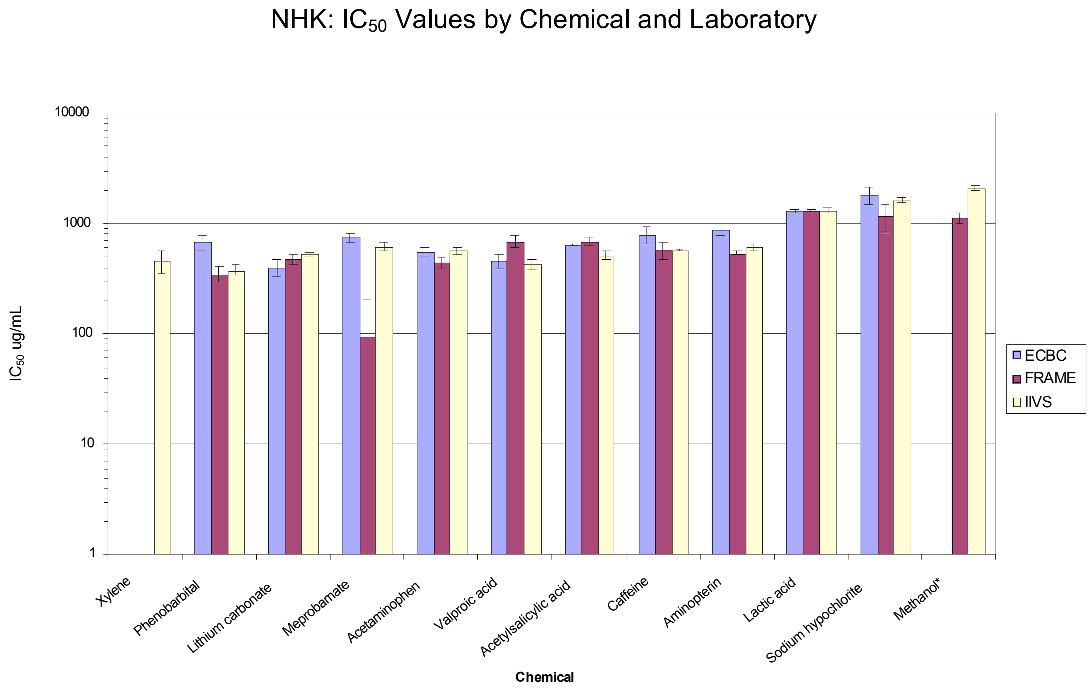

NHK: IC_{50} Values by Chemical and Laboratory
IC_{50} ug/mL
1000
100
10
1
ECBC
FRAME
IIVS
Propranolol*
Sodium fluoride
5-Aminosalicylic acid
Strychnine
Paraquat
Verapamil HCl
Phenol
Atropine sulfate
Carbamazepine
Epinephrine bitartrate
Nicotine
Physostigmine
Chemical

d


NHK: IC_{50} Values by Chemical and Laboratory
IC_{50} ug/mL
1000
100
10
1
ECBC
FRAME
IIVS
Chloral hydrate
Diethyl phthalate
Glutethimide
Cupric sulfate pentahydrate
Busulphan
Sodium oxalate
Phenylthiourea
Chloramphenicol
Disulfoton*
Trichloroacetic acid
Citric acid*
Boric acid
Chemical

e



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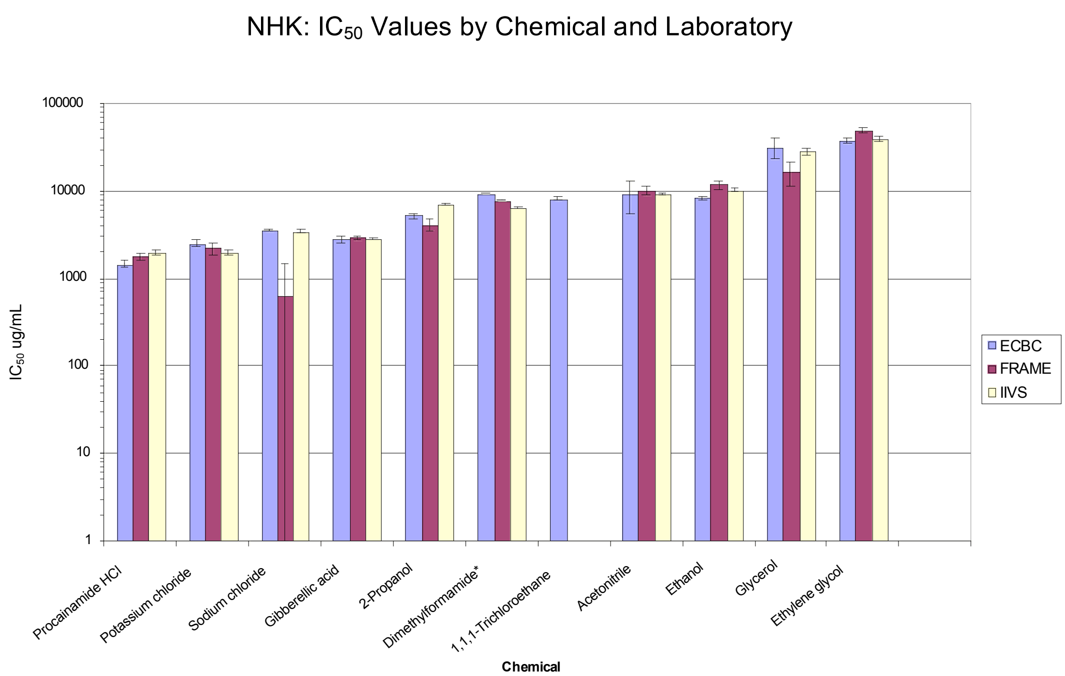


*Represents a chemical where the standard ANOVA indicates a significant difference in IC_{50} values between laboratories. Bars represent mean IC_{50} from each laboratory in μg/mL. Log IC_{50} values used to allow multiple data sets on each graph. Error bars show the standard deviation.

Table 5-4 Comparison of 3T3 and NHK IC_{50} Geometric Means

Reference Substance	3T3 NRU Test Method Geometric Mean[1] IC_{50} (µg/mL)	NHK NRU Test Method Geometric Mean[1] IC_{50} (µg/mL)	Difference (Orders of Magnitude)
Carbon tetrachloride	NA	NA	NA
Methanol	NA	1529[b]	NA
Aminopterin	0.006	669	5
Triphenyltin hydroxide	0.017	0.010	0
Colchicine	0.034	0.007	1
Cycloheximide	0.187	0.073	1
Triethylenemelamine	0.272	1.85	1
Cadmium II chloride	0.518	1.84	1
Sodium dichromate dihydrate	0.587	0.721	0
Sodium arsenite	0.759	0.477	0
Arsenic trioxide	1.96	5.26	0
Mercury II chloride	4.12	5.80	0
Hexachlorophene	4.19	0.029	2
Thallium I sulfate	5.74	0.152	1
Haloperidol	6.13	3.36	0
Endosulfan	6.35	2.13	0
Amitriptyline HCl	7.05	8.96	0
Diquat dibromide monohydrate	8.04	4.48	0
Propranolol	13.9	35.3	0
Dichlorvos	17.7	10.7	0
Paraquat	20.1	61.6	0
Fenpropathrin	24.2	2.43	1
Physostigmine	25.8	88.5	0
Propylparaben	26.1	16.6	0
Sodium selenate	29.0	10.2	0
Potassium cyanide	34.6	29.0	1
Verapamil HCl	34.9	66.5	0
Parathion	37.4	30.3	0
Sodium oxalate	37.7	337	1
Sodium lauryl sulfate (SLS)*	***41.7***	***3.99***	***1***
Cupric sulfate pentahydrate	42.1	197	1
Acetaminophen	47.7	518	1
Dibutyl phthalate	49.7	28.7	0
Epinephrine bitartrate	59.0	87.4	0
Phenol	66.3	75.0	1
Atropine sulfate	76.0	81.8	0
Busulfan	77.7	260	1
Sodium I fluoride	78	49.8	0
Phenylthiourea	79.0	336	1
Carbamazepine	103	83.2	1
Diethyl phthalate	107	120	0
Lindane	108	18.7	1
Chloramphenicol	128	348	0
Disulfoton	133	270	0
Caffeine	153	638	0
Strychnine	158	62.5	1
Glutethimide	174	174	0

Table 5-4 Comparison of 3T3 and NHK IC_{50} Geometric Means

Reference Substance	3T3 NRU Test Method Geometric Mean[1] IC_{50} (µg/mL)	NHK NRU Test Method Geometric Mean[1] IC_{50} (µg/mL)	Difference (Orders of Magnitude)
Chloral hydrate	183	133	0
Nicotine	361	107	0
Procainamide HCl	441	1741	1
Digoxin	466	0.001	5
Meprobamate	519	357	0
Lithium I carbonate	562[a]	468	0
Phenobarbital	573	448	0
Acetylsalicylic acid	676	605	0
Xylene	721[a]	466[a]	0
Citric acid	796	400	0
Trichloroacetic acid	902	413	0
Valproic acid	916	512	0
Sodium hypochlorite	1103	1502	0
5-Aminosalicylic acid	1667	46.7	2
Boric acid	1850	421	1
Lactic acid	3044	1304	0
Potassium I chloride	3551	2237	0
2-Propanol	3618	5364	0
Sodium chloride	4730	1997	0
Dimethylformamide	5224	7760	0
Ethanol	6523	10018	1
Gibberellic acid	7810[b]	2856	0
Acetonitrile	7951	9528	0
1,1,1-Trichloroethane	17248	8122[a]	1
Ethylene glycol	24317	41852	0
Glycerol	24655	24730	0

Table sorted by 3T3 IC_{50} values
[1]Laboratories combined; use of a geometric mean for the IC_{50} values in **Table 5-4** is consistent with the approach used for the RC millimole regression to obtain a single IC_{50} from multiple IC_{50} values (Halle 1998).
[a]Data available from only one laboratory
[b]Data available from only two laboratories
*Positive control (SLS) values (met acceptance criteria) from all test phases: N = 293 (3T3); N = 281 (NHK)
NA = not available

Two chemicals, digoxin and aminopterin, have IC_{50} values that differ by five orders of magnitude between the two cell types. Digoxin was much more toxic to the NHK cells and aminopterin was more toxic to the 3T3 cells. Hexachlorophene and 5-aminosalicylic acid IC_{50} values were different by two orders of magnitude and both were more toxic to the NHK cells than the 3T3 cells. The positive control (SLS) values for the two cell types differed by an order of magnitude (41.7 µg/mL for 3T3; 3.99 µg/mL for NHK). Of the IC_{50} reference substance values. 94.5% for both cell types were within at least 2 orders of magnitude of each other. **Table 5-5** illustrates the comparisons of the IC_{50} values.

Table 5-5 Difference in 3T3 and NHK NRU IC_{50} Values as Orders of Magnitude

Difference (Orders of Magnitude)	Percentage of Reference Substances
0	63.9% (46/72)
1	27.8% (20/72)
2	2.8% (2/72)
3	0
4	0
5	2.8% (2/72)
NA	2.8% (2/72)

Figure 5-3 RC IC_{50} Values vs 3T3 NRU IC_{50} Values for the 58 Common Chemicals

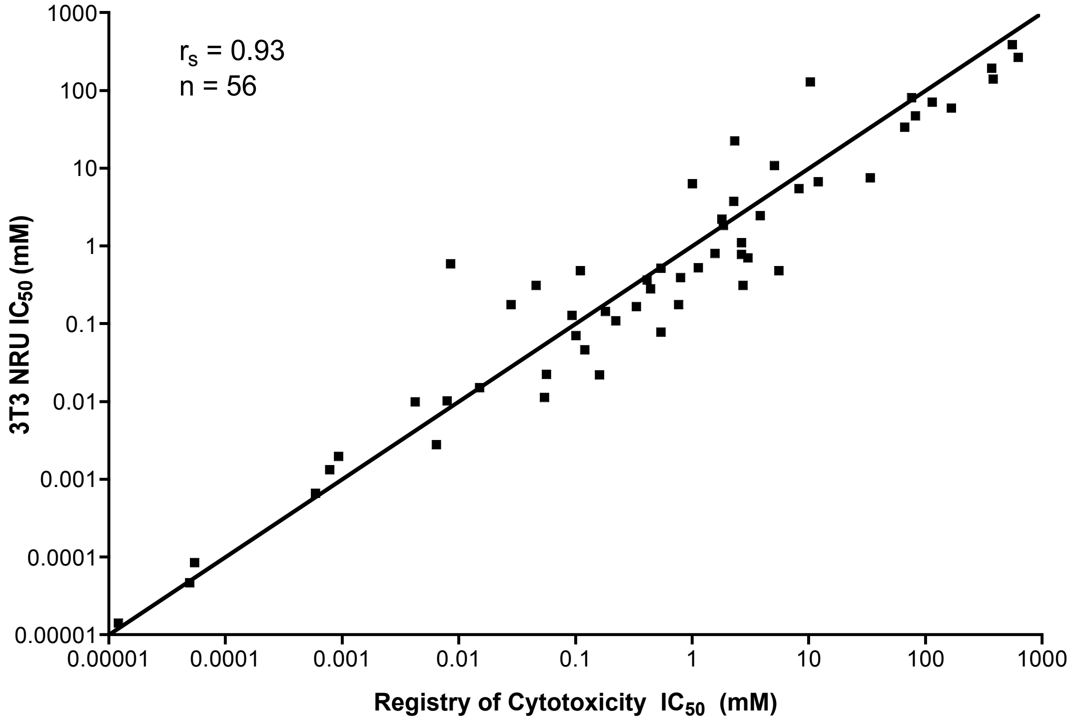
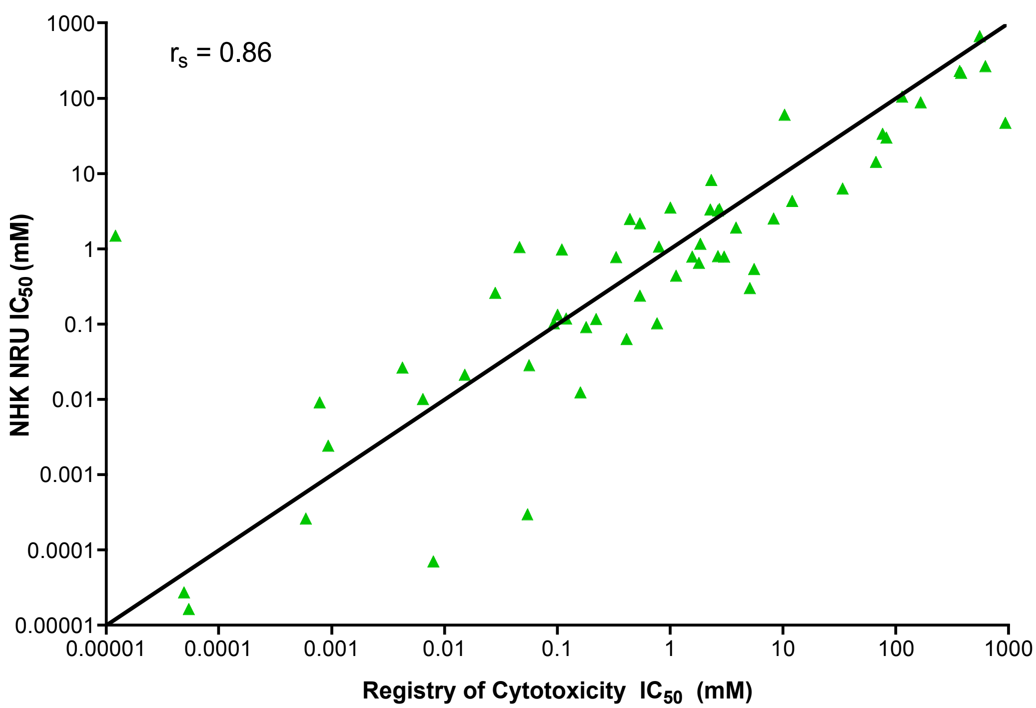


Figure 5-4 RC IC_{50} Values vs NHK NRU IC_{50} Values for the 58 Common Chemicals



5.5 Coded Reference Substances and GLP Guidelines

5.5.1 <u>Coded Reference Substances</u>

BioReliance acquired 73 high purity chemicals (72 reference substances and one positive control chemical, at 99% or greater purity when economically feasible) from reputable commercial sources (see **Appendix F**). BioReliance randomly coded each reference substance with a unique identification number when repackaging into multiple smaller units. These units were given an additional code unique for the respective cytotoxicity laboratories so that substances could be provided in a blinded fashion (see **Section 3.6** for distribution procedures). The reference substances were packaged and shipped such that their identities were concealed; however, all laboratories knew the identity of the positive control. The SMT revealed the reference substance codes for each phase after all laboratories had submitted their data and reports. Periodically, laboratories required additional aliquots of reference substance and BioReliance provided these aliquots from the original stock of reference substance in the same manner that the original aliquots were provided.

5.5.2 <u>Lot-to-Lot Consistency of Reference Substances</u>

One lot of each substance was purchased and each laboratory received aliquots from this same lot throughout the validation study. The substance suppliers provided certificates of analysis for each lot along with other chemical, physical, and safety information concerning the substance (e.g., MSDS documents).

5.5.3 <u>Adherence to GLP Guidelines</u>

BioReliance, ECBC, and IIVS, followed GLP procedures for all testing with the exception of tests designed to resolve technical challenges (e.g., formation of NR crystals, use of film plate sealers for volatile substances, slow growth of cells, etc.). These laboratories submitted data to their respective quality assurance unit (as per GLP requirements) and copies of the data were submitted to NICEATM. FAL followed most GLP guidelines, but their activities did not include independent quality assurance reviews of laboratory procedures or documentation. The Study Director for the FAL performed all data reviews and provided copies to NICEATM. Hard copy printouts of all data as well as electronic versions are available at NICEATM.

5.6 Study Timeline and NICEATM/ECVAM Study Participatory Laboratories

5.6.1 <u>Statement of Work (SOW) and Protocols</u>

The SMT provided the laboratories with an SOW prior to initiation of testing (see **Appendix G**) and proposed dates for completion of various aspects of the study (e.g., transfer of data, provision of reports, etc.). The SOW for the cytotoxicity laboratories defined the following:

- project objectives
- management and key personnel
- required facilities, equipment, and supplies
- quality assurance requirements
- test phases and schedules
- products (e.g., reports) required
- report preparation

The SOW for BioReliance contained all of the above and included requirements for:

- reference substance acquisition, preparation, and distribution
- solubility testing

The SMT, in consultation with the laboratories, prepared Test Method Protocols for each phase of the study. Cytotoxicity testing for each phase (in each laboratory) was initiated when the SMT received a signed protocol specific for that phase from the Study Director. Solubility testing for Phases I and II was performed prior to cytotoxicity testing for those phases while solubility testing for the Phase III substances was performed throughout Phases II and III.

5.6.2 Study Timeline

The actual timeline achieved in the study is shown in **Table 5-6**. The SMT eased the original timeline presented in the SOWs due to various factors (e.g., protocol revisions, side studies, acquisition of medium, etc.).

Table 5-6 Validation Study Timetable

	BioReliance	ECBC	FAL	IIVS
Receipt of SOW	Jun 2002	Jun 2002	Jun 2002	Jun 2002
Procurement of Chemicals	Jul 2002 - Jan 2003	NA	NA	NA
Solubility Testing	Jul 2002 - Jan 2003	Sep 2004	Dec 2003	Jan 2004
Distribution of Reference Substances Phase Ia Phase Ib Phase II Phase III	 Jul 2002 Sep 2002 Nov 2002 Feb - Mar 2003	NA	NA	NA
Initiation of Phase Ia	NA	Aug 2002	Aug 2002	Aug 2002
Completion of Phase Ia	NA	Nov 2002	Nov 2002	Oct 2002
Initiation of Phase Ib	NA	Dec 2002	Dec 2002	Dec 2002
Completion of Phase Ib	NA	May 2003	May 2003	May 2003
Initiation of Phase II	NA	Jun 2003	Jun 2003	Jun 2003
Completion of Phase II	NA	Nov 2003	Nov 2003	Nov 2003
Initiation of Phase III	NA	Dec 2003	Dec 2003	Dec 2003
Completion of Phase III	NA	Dec 2004	Dec 2004	Jan 2005

NA- not applicable; SOW = BioReliance distributed reference substances; ECBC, FAL, AND IIVS tested the reference substances

5.6.3 Participatory Laboratories

BioReliance Corporation
14920 Broschart Road
Rockville, Maryland 20850-3349
Study Director: Dr. Martin Wenk

U.S. Army Edgewood Chemical & Biological Center (ECBC)
Molecular Engineering Team
Aberdeen Proving Ground, MD 21010
Study Director: Dr. Cheng Cao

Institute for In Vitro Sciences (IIVS)
21 Firstfield Road Suite 220
Gaithersburg, MD 20878
Study Director: Mr. Hans Raabe

FRAME (Fund for the Replacement of Animals in Medical Experiments)
Alternatives Laboratory (FAL)
Queens Medical Centre
University of Nottingham
Nottingham NG7 2UH
United Kingdom
Study Director: Dr. Richard Clothier

5.7 Availability of Data

All data were submitted and provided to the SMT via electronic files and paper copies. The laboratories also maintained copies of all raw data and the electronic files.

5.8 Solubility Test Results

This study evaluated a solubility protocol (see **Section 2-7** and **Appendix B-3)** designed to identify the solvent that would provide the highest soluble concentration of a reference substance for *in vitro* testing. Each laboratory performed a solubility test on all reference substances. To avoid the use of different solvents by the laboratories when testing the same substance, the SMT assigned the solvents used for *in vitro* testing (see **Table 6-9**). The objectives of the solubility testing were to evaluate the utility and appropriateness of the solubility protocol and to evaluate the concordance among laboratories in the solvent selected for each of the 72 reference substances.

5.8.1 Solubility Data

BioReliance was the first laboratory to evaluate the solubility of the reference substances, first in media, then in DMSO, and then in ETOH at 400 and 200 mg/mL. Based on this experience, a solubility protocol for the *in vitro* laboratories was developed to test at lower test article concentrations and to test with the various solvents at concentrations that would be equivalent when applied to the cultures (see **Table 2-5**). The solubility flow chart (**Figure 2-7**) illustrates the tests for chemical solubility in medium, DMSO, and ETOH. **Table 5-7** provides the solubility results in mg/mL.

Table 5-7 Solubility Results (data presented in mg/mL)

Reference Substance	BioReliance[1]				SMT[2] Selection	ECBC[3]				FAL[3]				IIVS[3]			
	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH		3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH
Phase I																	
Arsenic III trioxide	0.25	0.05	< 2	< 2	Medium	***0.025[6]***	***0.025[6]***	***< 0.2***	***< 0.2***	***0.135[6]***	***0.135[6]***	***< 0.2***	***< 0.2***	***< 0.02[6]***	***< 0.02[6]***	***< 0.2***	***< 0.2***
Ethylene glycol	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Propranolol HCl	< 2	10	200	20	DMSO	0.2	2	200	NT	20	20	200	NT	20	2	NT	NT
Phase II																	
Aminopterin	2	2	NT	NT	DMSO	2.0	< 2	200	NT	< 2	2	200	NT	0.2	0.2	200	NT
Cadmium II chloride	< 2	< 2	200	< 200	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	< 0.2	< 0.2	20	< 20
Chloramphenicol	2	2	400	< 200	DMSO	2.0	< 2	200	NT	< 2	< 2	200	NT	0.2	0.2	20	20
Colchicine	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Lithium I carbonate	0.25	10	< 2	NT	Medium	0.2	2.0	< 20	< 20	0.2	2	< 200	< 200	0.2	2	< 2	< 2
Potassium I chloride	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
2-Propanol	400	400	400	400	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium I fluoride	20	20	< 200	< 200	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium selenate	200	200	< 200	< 200	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Phase III																	
Acetaminophen	10	10	400	< 200	DMSO	2	2	NT	NT	2	2	NT	NT	< 2	< 2	200	NT
Acetonitrile	400	400	400	400	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Acetylsalicylic acid	10	10	400	200	DMSO	2	2	NT	NT	< 2	< 2	200	NT	2	2	NT	NT
5-Aminosalicylic acid	2	2	< 200	< 200	Medium	2	2	NT	NT	2	2	NT	NT	2	2	NT	NT
Amitriptyline HCl	200	200	NT	NT	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	0.2	0.2	200	NT

Table 5-7 Solubility Results (data presented in mg/mL)

Reference Substance	BioReliance[1]				SMT[2] Selection	ECBC[3]				FAL[3]				IIVS[3]			
	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH		3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH
Atropine sulfate	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Boric aid	40	40	200	< 200	Medium	20	20	NT	NT	20	20	NT	NT	2	2	NT	NT
Busulfan	< 2	< 2	40	< 200	DMSO	< 2	< 2	200	NT	< 2	< 2	50[6]	< 200	< 0.2	< 0.2	20	< 200
Caffeine	10	10	20	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Carbamazepine	< 2	< 2	40	< 200	DMSO	0.2	0.2	20	20	< 2	< 2	200	NT	< 0.2	< 0.2	2	< 20
Carbon tetrachloride	2	10	NT	NT	DMSO	20	20	NT	NT	< 0.2	< 0.2	2	NT	20	20	NT	NT
Chloral hydrate	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Citric acid	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Cupric sulfate pentahydrate	1	0.5	< 2	2	Medium	2	0.2	< 200	< 200	2	2	NT	NT	0.2	0.2	< 200	NT
Cycloheximide	20	20	400	< 200	Medium	20	20	NT	NT	20	20	NT	NT	2	2	NT	NT
Dibutyl phthalate	< 2	< 2	400	400	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	< 2	< 2	200	NT
Dichlorvos	10	10	NT	NT	DMSO	2	2	NT	NT	< 2	< 2	200	NT	2	2	NT	NT
Diethyl phthalate	< 2	< 2	400	400	DMSO	< 2	< 2	200	NT	0.2	< 0.2	200	NT	< 2	< 2	200	NT
Digoxin	0.05	0.05	200	< 200	DMSO	< 2	< 2	200	NT	< 0.2	< 0.2	200	NT	< 2	< 2	200	NT
Dimethylformamide	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Diquat dibromide monohydrate	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Disulfoton	< 2	< 2	500	NT	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	< 2	< 2	200	NT
Endosulfan	< 0.05	< 0.05	40	NT	DMSO	< 0.2	< 0.2	20	< 200	< 0.2	< 0.2	2	< 200	< 0.2	< 0.2	20	< 200
Epinephrine bitartrate	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	2	2	NT	NT
Ethanol	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Fenpropathrin	< 20	< 20	500	NT	DMSO	< 2	< 2	200	NT	< 0.2	< 0.2	200	NT	< 2	< 2	200	NT

Table 5-7 Solubility Results (data presented in mg/mL)

Reference Substance	BioReliance[1]				SMT[2] Selection	ECBC[3]				FAL[3]				IIVS[3]			
	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH		3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH
Gibberellic acid	10	10	NT	NT	Medium	2	2	NT	NT	2	2	NT	NT	2	2	NT	NT
Glutethimide	< 2	< 2	500	NT	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	< 2	< 2	200	NT
Glycerol	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Haloperidol	< 20	< 20	40	NT	DMSO	< 0.2	< 0.2	20	< 20	< 0.2	< 0.2	20	< 20	< 2	< 2	20	< 20
Hexachlorophene	0.05	< 0.05	400	400	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	< 2	< 2	200	NT
Lactic acid	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Lindane	< 0.05	< 0.05	400	< 200	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	< 0.2	< 0.2	20	< 200
Meprobamate	1	1	200	NT	DMSO	2	2	200	NT	2	2	200	NT	< 0.2	< 0.2	200	NT
Mercury II chloride	0.125	0.125	400	< 200	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	< 0.2	< 0.2	200	NT
Methanol	40	40	400	400	DMSO	20	20	NT	NT	20	20	NT	NT	< 2	< 2	200	NT
Nicotine	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Paraquat	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Parathion	0.05	< 0.05	400	400	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	< 2	< 2	200	NT
Phenobarbital	2	2	200	< 200	DMSO	2	2	NT	NT	< 2	< 2	200	NT	< 2	< 2	200	NT
Phenol	40	40	400	400	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Phenylthiourea	2	2	400	< 200	DMSO	2	< 2	200	NT	20	20	NT	NT	< 2	< 2	200	NT
Physostigmine	2	2	400	200	DMSO	2	2	NT	NT	< 2	< 2	200	NT	< 2	< 2	200	NT
Potassium cyanide	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Procainamide HCl	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Propylparaben	0.25	0.25	400	400	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	< 2	< 2	200	NT
Sodium arsenite	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT

Table 5-7 Solubility Results (data presented in mg/mL)

Reference Substance	BioReliance[1]				SMT[2] Selection	ECBC[3]				FAL[3]				IIVS[3]			
	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH		3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH
Sodium chloride	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium dichromate dihydrate	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium hypochlorite	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium oxalate	< 0.05	20	0.125	< 0.05	Medium	< 0.2	20	0.2	< 2	20	20	NT	NT	***< 0.2***	***< 0.2***	***< 0.2***	***< 0.2***
Strychnine	< 2	< 2	2	2	Medium	0.2	< 0.2	2	2	0.2	0.2	< 200	< 200	***< 0.2***	***< 0.2***	***< 0.2***	***< 0.2***
Thallium I sulfate	1	0.5	< 2	< 2	Medium	0.2	0.2	< 200	< 200	***< 0.2***	***< 0.2***	***< 0.2***	***< 0.2***	0.2	0.2	< 20	< 200
Trichloroacetic acid	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
1,1,1-Trichloroethane	10	10	400	400	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Triethylenemelamine	< 2	< 2	2	< 20	DMSO	0.2	0.2	< 200	< 200	< 0.2	< 0.2	2	< 2	***< 0.2***	***< 0.2***	***< 0.2***	***< 0.2***
Triphenyltin hydroxide	< 0.05	< 0.05	10	< 20	DMSO	< 0.2	< 0.2	2	< 20	< 0.2	< 0.2	2	< 200	< 2	< 2	2	< 20
Valproic acid	10	2	NT	NT	DMSO	2	2	NT	NT	< 2	< 2	200	NT	2	< 2	200	NT
Verapamil HCl	< 0.05	0.25	200	NT	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	< 0.2	< 0.2	20	NT
Xylene	1	1	500	NT	DMSO	< 2	< 2	200	NT	2	< 2	200	NT	< 2	< 2	200	NT

Table sorted by study phase and alphabetical by reference chemical

[1]Used a different solubility protocol from the *in vitro* cytotoxicity laboratories.

[2]Solvents selected by the SMT for cytotoxicity testing. BioReliance results were used to determine solvents for Phases I and II. Results from all laboratories were used to determine solvents for Phase III. Media were treated as one result. If insoluble in one medium and soluble in DMSO, DMSO was selected.

[3]Used protocol in **Figure 2-7**.

[4]Dulbecco's Modification of Eagle's Medium.

[5]Keratinocyte Growth Medium (KGM® from CAMBREX Clonetics®).

[6]Protocol deviation.

In vitro laboratories agreed on solvent. *In vitro* laboratories did not agree on solvent. ***bold*** Protocol did not provide enough information to select a solvent.

NT– not tested.

5.8.2 Solubility Effects on the *In Vitro* NRU Cytotoxicity Test Method Data

The laboratories reported solubility results for the stock solutions for each 3T3 and NHK NRU test. Prior to the additions of the NR dye medium for the NRU test method, the laboratories visually observed the test cultures and documented noticeable precipitate found in the test plates. **Table 5-8** illustrates the existence of solubility issues (in both 3T3 and NHK NRU experiments) as evidenced by the observation of precipitates with some reference substances. Volatility difficulties, indicated by the use of film plate sealers during substance incubation, are also indicated in this table. **Section 3.5** provides additional information on the solubility of specific reference substances.

Table 5-8 Reference Substances with Precipitate (PPT) and Volatility Issues[1]

Reference Substances	3T3 NRU Test Method				NHK NRU Test Method			
	PPT 2X Stock Dilutions	PPT 1X Plate Dilutions	PPT Stock and Plate Dilutions	Volatility	PPT 2X Stock Dilutions	PPT 1X Plate Dilutions	PPT Stock and Plate Dilutions	Volatility
Acetonitrile				X				X
Aminopterin		X			X			
5-Aminosalicylic acid	X							
Arsenic III trioxide	X				X			
Cadmium II chloride		X					X	
Carbamazepine			X					
Carbon tetrachloride			X		X			
Citric acid						X		
Cupric sulfate pentahydrate						X		
Dibutyl phthalate		X					X	
Dichlorvos				X				X
Diethyl phthalate	X						X	
Digoxin			X					
Dimethylformamide						X		
Disulfoton			X				X	
Endosulfan	X			X				X
Ethanol				X				X
Fenpropathrin			X				X	
Gibberellic acid	X				X			
Glutethimide					X			
Lindane			X	X			X	
Lithium I carbonate	X				X			
Nicotine				X				X
Parathion	X						X	
Phenol				X				X
Potassium I chloride		X						
Potassium cyanide		X		X				X
2-Propanol				X				X
Sodium arsenite		X						X
Sodium chloride						X		
Sodium I fluoride		X				X		
Sodium hypochlorite				X				
Sodium oxalate			X			X		
Strychnine	X				X			
Trichloroacetic acid						X		
1,1,1-Trichloroethane	X						X	
Valproic acid	X							
Verapamil HCl					X			
Xylene	X				X			

Table sorted alphabetical by reference substance

[1]Results are based on at least one laboratory having precipitate and volatility issues with a substance. Volatility was denoted by the use of plate sealers during testing. 2X stock dilutions are prepared for each of 8 test substance concentrations. 1X plate dilutions are the result of diluting the 2X stock solutions with medium in the 96-well plate.

5.9 Summary

- Modifications and revisions made to the protocols during Phases I and II contributed to the optimization of the final protocols used in Phase III of the study. The changes did not have a negative impact on the 3T3 and NHK NRU test method data. Generally, changes enhanced the performance of the *in vitro* NRU cytotoxicity test methods and allowed more tests to meet acceptance criteria.
- FAL improved testing quality by modifying the methods used to propagate the NHK cells prior to Phase II testing. Positive control IC_{50} data in Phases II and III from FAL more closely resembled the data from the other laboratories after test methods were optimized.
- Summary test data are presented in tabular and graphical formats. Comparisons of 3T3 IC_{50} values to NHK IC_{50} values show that most values (92%) are within one order of magnitude of each other. Digoxin and aminopterin data had a difference of five orders of magnitude when IC_{50} values are compared between the cell types.
- The BioReliance, ECBC, and IIVS laboratories performed the 3T3 and NHK NRU experiments in compliance with GLP guidelines and submitted quality data. The reference substance quality was maintained throughout the study and lot-to-lot consistency was not a factor in testing.
- Each laboratory followed the same solubility protocol when making reference substance dilutions yet differences in results were present. Judgment of solubility is subjective (as per this protocol).

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